CLINICAL MYCOLOGY

Oxygen requirements of *Aspergillus* species

LINDA A. HALL* and D. W. DENNING*†

* Department of Medicine, University of Manchester, Hope Hospital, Salford and † Department of Infectious Diseases and Tropical Medicine (Monsall Unit), North Manchester General Hospital, Crumpsall, Manchester

Summary. The growth of 24 *Aspergillus* isolates at low oxygen tensions was assessed. Isolates selected included *A. fumigatus* (10), *A. terreus* (6), *A. niger* (6), *A. nidulans* (1) and *A. flavus* (1). Three different agar media were used—potato dextrose agar (PDA), pH 5·6; brain heart infusion (BHI), pH 7·4; and a specially developed medium (Hall's) containing resazurin—with oxygen concentrations of 0, 0·025, 0·1, 0·5 and 2·5 %. The CO₂ concentration was 5 %. Agar plates were inoculated with 2×10^7 conidia/ml, loaded into jars and flushed with a special gas mixture at 37°C. The plates were inspected at intervals of 3, 5 and 10 days. On Hall's medium, none of the isolates grew at oxygen concentrations of 0 or 0·025 %, but 21 (88 %) of 24 grew at 0·1 %. On PDA and BHI, all 14 isolates tested grew at oxygen concentrations of 0·5 and 2·5 %. Three of these 14 conidiated on PDA at oxygen 0·5 % and 12 of 14 conidiated on PDA at oxygen 2·5 %. None grew without oxygen on these media. Thus, pathogenic *Aspergillus* spp. are capable of growth at low oxygen tensions, and this may have implications for pathogenicity and antifungal activity.

Introduction

Aspergilli are ubiquitous throughout the environment and are found in organic debris, decaying vegetation and soil.¹ As causes of clinical infection, *Aspergillus* spp. are predominantly respiratory pathogens in immunocompromised hosts, but different host groups may also be susceptible to specific non-pulmonary disease (e.g., kidney disease in diabetic patients).² Invasive aspergillosis is rare in immunocompetent hosts. The increase of immunocompromised patients and the greater degree of immune suppression within this group are responsible for a substantial increase in the incidence of this disease. As a proportion of all systemic mycoses found at autopsy, invasive aspergillosis now comprises 60 %, as compared to 17 % a decade ago.³ The past decade has seen the emergence of new clinical entities caused by *Aspergillus* spp. and new host groups developing different forms of aspergillosis (e.g., pulmonary aspergillosis in patients with AIDS).⁴ Invasive aspergillosis is a devastating opportunist fungal disease with an unacceptably high mortality rate (in allogeneic bone marrow transplant patients and cerebral aspergillosis it exceeds 94 %).⁵ It is difficult to diagnose infection in time for effective treatment to be given.

The most important single factor allowing anaerobic or micro-aerophilic organisms to flourish in body tissues is a low oxygen tension, which is usually the result of a compromised blood supply and poor tissue

perfusion. Typically, the pathological appearance of invasive aspergillosis shows infarcted tissue invaded by fungal hyphae.⁶ The aim of this study was to mimic these deep tissue conditions and establish the minimum oxygen requirements for growth of *Aspergillus* spp.

Materials and methods

Aspergillus isolates

Twenty-four *Aspergillus* isolates were selected: *A. fumigatus* (10), *A. terreus* (6), *A. niger* (6), *A. nidulans* (1) and *A. flavus* (1); most were clinical isolates. The inocula were prepared by culturing each isolate on

Fig. 1. Individual stacking ring with petri dish in position. A, inlet port; B, outlet port; C, peripheral seal; D, base seal.

Received 8 April 1994; revised version accepted 28 May 1994.

† Correspondence should be sent to Dr D. W. Denning.

Fig. 2. Close-up of stacking insert and gas manifold. A, gas inlet manifold.

Fig. 3. Anaerobic jar showing modifications and accessories. A, sealed quick container (non-return inlet valve); B, pressure-relief valve; C, plate stacking insert with gas manifold; D, plate ejector or petri dish ejector; E, petri dish installing plate.

Potato Dextrose Agar (PDA; Oxoid/Unipath) for 10 days at 37°C. Conidia were collected in sterile PBS-Tween (phosphate-buffered saline 0.9% containing Tween 80 0.01%; Sigma) and stored at 4°C. Each inoculum comprised 2×10^7 conidia/ml quantified by counting in a haemocytometer.

Media

For testing, Brain Heart Infusion (BHI) Agar (Unipath), pH 7.4, PDA, pH 5.6, and a modified

defined medium (Hall's), pH 5, were used. Hall's medium contained (/L of distilled water): glucose 10 g, yeast extract (Oxoid) 10 g, NaHCO_3 6 g, L-cysteine HCl (Sigma) 1 g, K_2HPO_4 0.06 g, KH_2PO_4 0.06 g, $(\text{NH}_4)_2\text{SO}_4$ 0.12 g, NaCl 0.12 g, MgSO_4 0.012 g and CaCl_2 0.012 g. The pH was adjusted to 5 with concentrated HCl. Resazurin (Sigma) 0.02 g/L and agar (Oxoid) to 1.8% were added. Resazurin is a reducing agent that is decolourised at low redox potential (e.g., ≤ -42 mV).⁷ The criterion for absolute anaerobiosis that is usually accepted for culture purposes is that a buffered resazurin solution at pH 7 remains colourless. Hall's medium was colourless at pH 5 before and after autoclaving. The medium was heated to dissolve the agar and bubbled with CO_2 to remove free O_2 . This was confirmed by a colour change from red to colourless of the resazurin indicator. It was then autoclaved at 115°C for 10 min and the lid was kept tightly closed. The medium remained colourless, at pH 5 before and after autoclaving. The molten agar was then poured into petri dishes (previously stored in an anaerobic chamber) and allowed to cool.

Incubation atmospheres

The oxygen concentrations used were 0, 0.025, 0.1, 0.5 and 2.5%. The CO_2 concentration was 5%. Gases were obtained from the British Oxygen Company, Morden and had quality assurance certificates. Specially adapted anaerobic jars were constructed (figs. 1–4), modified to ensure that the gas flow was uniform within each jar. Each agar plate was contained in two rubber O-Seal rings between which gas flowed. The gas was piped in and flowed evenly to each ring.

The poured plates were left in the anaerobic chamber for 2–5 days, then each was inoculated with 2×10^7 conidia with aseptic techniques in an atmosphere of N_2 85%, CO_2 10% and H_2 5%. Plates were sealed with paraffin and loaded into the modified anaerobic jars, transferred to a hot room at 37°C and continuously flushed with the special gas mixtures at a flow rate of 1 L/min (fig. 4). The plates were inspected at intervals of 3, 5 and 10 days in the anaerobic chamber and the presence or absence of growth was recorded. Plates that had shown no growth after 10 days were transferred to air at 37°C as a control to confirm a viable inoculum.

Results

The initial experiments were conducted with 14 isolates and PDA and BHI media at oxygen concentrations of 0, 0.5 and 2.5%. None of the isolates grew at an oxygen concentration of 0% on either medium but all grew on transfer to air at 37°C. All 14 isolates grew on PDA and BHI at oxygen 0.5% and 2.5%; three of them conidiated in oxygen 0.5% and 12 of

Fig. 4. Equipment *in situ*. A, high-pressure gas cylinder with specified gas mixture; B, high-pressure output regulator; C, flowmeter; D, anaerobic jar.

Table I. Growth of *Aspergillus* spp. on PDA and BHI at increasing oxygen concentrations

Species	Isolate no.	Growth and conidiation (C) at oxygen concentration					
		0%		0.5%		2.5%	
		PDA	BHI	PDA	BHI	PDA	BHI
<i>A. fumigatus</i>	10	—	—	+	+	+C	+
	11	—	—	+C	+	+C	+
	20	—	—	+C	+	+C	+
	41	—	—	+	+	+C	+
	71	—	—	+C	+	+C	+
	72	—	—	+	+	+C	+
<i>A. terreus</i>	8	—	—	+	+	+C	+
	10	—	—	+	+	+C	+
	18	—	—	+	+	+C	+
<i>A. niger</i>	1	—	—	+	+	+	+
	4	—	—	+	+	+C	+
	6	—	—	+	+	+C	+
<i>A. nidulans</i>	2	—	—	+	+	+	+
<i>A. flavus</i>	14	—	—	+	+	+C	+

—, no growth by day 10; +, growth by day 3; +C, growth and conidiation by day 3.

them at 2.5% on PDA (table I). Growth was quite evident by day 3 and appeared to be more vigorous on PDA than BHI.

Hall's medium was then developed because of uncertainty about the concentration of dissolved oxygen in the standard media. Hall's medium was tested at oxygen 0.5% and by day 3 there was some change from colourless to purple. This colour change represents increasing dissolved oxygen concentration. Therefore, Hall's medium was tested at lower oxygen concentrations (0.025 and 0.1%). Each position in

each jar was tested and no consistent variation in time to colour change was detected in any position in the jar.

On Hall's medium, there was no apparent growth on any plate by day 10 at oxygen 0.025% (table II). In oxygen 0.1%, 16 of 24 isolates showed varying degrees of growth by day 3. By day 5, 20 of 24 isolates had grown and 21 of 24 grew by day 10. None of these isolates conidiated at oxygen 0.1% on this medium.

Plates that exhibited no growth on Hall's medium after 10 days were incubated in air at 37°C and then at

Table II. Growth of *Aspergillus* spp. on Hall's medium at low oxygen concentrations at day 3 and day 10

Species	Isolate no.	Growth at oxygen concentration					
		0 %		0.025 %		0.1 %	
		3	10	3	10	3	10
<i>A. fumigatus</i>	10	—	—	—	—	+	+
	11	—	—	—	—	+	+
	15	—	—	—	—	+	+
	20	—	—	—	—	+	+
	41	—	—	—	—	+	+
	49	—	—	—	—	+	+
	50	—	—	—	—	—	+
	51	—	—	—	—	—	—
	71	—	—	—	—	+	+
	72	—	—	—	—	+	+
<i>A. terreus</i>	4	—	—	—	—	+	+
	6	—	—	—	—	—	—
	8	—	—	—	—	+	+
	10	—	—	—	—	+	+
	17	—	*	*	*	—	+
	18	—	—	—	—	—	+
<i>A. niger</i>	1	—	—	—	—	—	+
	4	—	—	—	—	+	+
	6	—	—	—	—	+	+
	8	—	—	—	—	—	+
	9	—	—	—	—	+	+
	10	—	—	—	—	—	—
<i>A. nidulans</i>	2	—	—	—	—	+	+
<i>A. flavus</i>	14	—	—	—	—	+	+

—, no growth; +, growth.

* Bacterial contamination.

4°C. All isolates subsequently grew either at 37°C (22 of 24) or at 4°C (2 of 24). This indicated that despite 10 days of oxygen deprivation and otherwise ideal germination conditions, the conidia were still viable and capable of germination.

Discussion

A new medium and test system were developed to assess the oxygen requirements of pathogenic *Aspergillus* spp. with resazurin as an internal control for anaerobic conditions. The equipment was tested with the resazurin as an indicator dye and the positioning of the agar plates in the jars made no difference to the results. The concentration of oxygen was uniform throughout the jars and surface of the agar. The time taken for colouration of the medium was consistent with the different oxygen concentrations used— ≥ 5 days at 0.025 % and ≤ 3 days at 0.5 %. The system

with medium at constant pH and set up in anaerobic conditions decolourised the resazurin. This indicator changes to purple as the redox potential rises, which would occur before any substantial rise in dissolved oxygen concentration.⁸ Thus, surface oxygen concentration would have been higher than agar oxygen concentrations initially. Similar or identical oxygen concentrations in gas and agar are likely to have been achieved within 48–72 h of setting up the equipment, as judged by a medium colour change at higher oxygen concentrations, and possibly earlier at lower oxygen concentrations. Therefore, there would have been much oxygen flux in the system initially, but not over the latter part of the experimental period.

Pathogenic *Aspergillus* spp. appear to be capable of growth at very low oxygen concentrations. However, they are clearly not capable of anaerobic growth as defined by growth at oxygen < 0.01 %.⁹ The growth rate is slowed at low oxygen concentrations and conidiation is delayed or absent. Conidiation varied with the medium used. Isolates that did not grow under experimental conditions subsequently grew when incubated in air, indicating that a low oxygen tension is not harmful to the conidia of *Aspergillus* spp. Thus oxygen can be considered an essential element for the growth but not survival of *Aspergillus* spp. These experiments did not allow determination of the absolute minimum oxygen requirement for growth.

The ability of aspergilli to continue to grow, albeit slowly, at low oxygen tensions has implications for pathogenicity. *Aspergillus* spp. are primarily pulmonary pathogens but different clinical and radiological patterns of disease are seen. Aspergilli often spread to the brain causing cerebral infarction. Different patterns of invasive aspergillosis could reflect differences between isolates in oxygen requirements, although only minor variation was detected in the isolates we tested. This variation might have clinical significance, although the actual concentrations of oxygen in infarcted tissue in invasive aspergillosis are not known. Other determinants of pathogenicity are likely to affect clinical patterns of aspergillosis and low oxygen concentration may affect, for example, the secretion of extracellular virulence factors. Whether susceptibility to antifungal agents varies at different oxygen concentrations is not known.

We are indebted to the Medical Physics Department, Hope Hospital, for constructing the agar stacking and gas flow arrangements in the anaerobic jars, and also to the Microbiology Department at Hope Hospital for the use of their facilities, to Dr Michelle Lawrence for her advice and helpful suggestions and to Mrs Julie Young for typing the manuscript.

References

1. Bardana EJ. The clinical spectrum of aspergillosis. Part I. Epidemiology, pathogenicity, infection in animals and immunology of *Aspergillus*. *CRC Crit Rev Clin Lab Sci* 1980; **13**: 21–83.
2. Bodey GP, Vartivarian S. Aspergillosis. *Eur J Clin Microbiol Infect Dis* 1989; **8**: 413–437.
3. Groll A, Shah PM, Menzel C, Just G, Schneider M, Hübner K. Invasive mycosis in post-mortem findings. *Trends in Invasive Fungal Infections II*, Manchester, September 2–4, 1993; abstract no. 1.
4. Denning DW, Follansbee S, Scolaro M, Norris S, Edelstein D, Stevens DA. Pulmonary aspergillosis in AIDS. *N Engl J Med* 1991; **324**: 654–662.
5. Denning DW, Stevens DA. Antifungal and surgical treatment

- of invasive aspergillosis: review of 2,121 published cases. *Rev Infect Dis* 1990; **12**: 1147–1201.
6. Denning DW, Ward PN, Fenelon LE, Benbow EW. Lack of vessel wall elastolysis in human invasive pulmonary aspergillosis. *Infect Immun* 1992; **60**: 5153–5156.
7. Visser W, Scheffers WA, Battenburg-Van Der Vegte WH, Van Dijken JP. Oxygen requirements of yeasts. *Appl Environ Microbiol* 1990; **56**: 3785–3792.
8. Wimpenny JWT. The effect of E_h on regulatory processes in facultative anaerobes. *Biotech Bioengin* 1969; **11**: 623–629.
9. Park MK, Myers RAM, Marzella L. Oxygen tensions and infections: Modulation of microbial growth, activity of antimicrobial agents, and immunologic responses. *Clin Infect Dis* 1992; **14**: 720–740.
-